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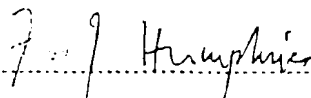
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EP 0 287 075 B1

Description

The description relates to a method of producing IFN beta in an animal host cell. The IFN beta isolated with high yield from the cell supernatant has a specific activity of about 3.8×10^8 IU/mg protein. Biological and immunological tests show that the isolated IFN-beta is substantially identical with natural IFN-beta. The IFN-beta produced by the method described is 95% glycosylated, and the structure and sequence of glycosylation is substantially similar to but not identical with that of natural IFN-beta.

Interferons are a group of antivirally active polypeptides formed through contact with exogenous inductors (e.g. viruses, nucleic acids or certain antigens) of the cells in question. One sub-group, beta-interferons (IFN-beta), are formed mainly by fibroblasts, Havell et al (1972) and Stewart II (1979). At present there are two known species of beta-interferons. Their immunological properties are similar, so that monoclonal neutralising antibodies can be isolated which activate both interferons similarly - see Zilberstein et al (1985). On the other hand there is no cross-hybridisation between IFN-beta-2 mRNA and samples of IFN-beta-1 cDNA in RNA gel blot hybridisation experiments or vice versa, Sehgal et al (1980).

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IFN-beta-1 from human diploid fibroblasts (FS4),
hereinafter called IFN-beta, has been in clinical
use for some time. As early as 1983 it was licensed
by the German Federal Office of Health for treatment
of severe life-threatening virus infections. Owing
to its efficiency and the absence of other wide-
spectrum virostatics, it has become the preferred
substance in many cases.

However, clinical use of the drug is greatly
restricted by the high price, since production on
the basis of normal non-transformed diploid
fibroblasts requires use of rare, expensive raw
materials and of cost-intensive cell substrates, and
since there are narrow limits to the extent to which
the processing operations can be rationalised.

This situation very quickly stimulated a search for
alternative production methods, and was one of the
most important motives for the development of modern
genetic-engineering methods in general. Inclusion
of the gene for human IFN-beta in heterologous host
cell systems was obviously the only true alternative
to conventional optimisation processes, since their
prospects of success appeared greatly restricted by
physiological barriers.

Out of the three main available groups of host cell
systems, i.e. procaryotes, lower and higher
eucaryotes, the general preference was initially for
the bacterial host system Escherichia coli (E.coli)

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on the basis of the prior art and the extremely low
production costs.

In spite of the high expectations regarding this
production system, it has not hitherto been possible
to process the large amounts of crude IFN-beta
obtainable by this method on an industrial scale,
Taniguchi et al (1980) and Goeddel (1980). This
difficulty is mainly due to the fact that the raw
material is present in the form of denatured
inclusion bodies in the host cell. This enables the
constituents of the host cell to be efficiently
separated, but hitherto the yield of clinically
useful material has been very low owing to the
problems of subsequent processing (difficult
solubility, occurrence of incorrect sulphur-bridge
bonds). There is a partial lack of activity
probably due to conformation changes as a result of
denaturing or incorrectly formed intrachenar sulphur
bridges, Lawn et al (1981), and additionally the
product, in contrast to the naturally occurring
form, is not glycosylated.

In this situation it has become increasingly
necessary to push forward with the development of
eucaryotic expression systems whereby even
heterologous gene products of more complex structure
can be produced in substantially authentic form by
correct "processing". Systems of this kind have
been described by a number of authors over the
years, Reyes et al (1982), Mitrani-Rosenbaum et al
(1983), Smith et al (1983), McCormick et al (1984),

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Chernajovsky et al (1984), Fukunaga et al (1984),
Page et al (1985).

Basic construction

We shall now describe the construction of a new BIC
cell line by genetic-engineering methods whereby
large quantities, compared with conventional
methods, of a native product substantially identical
with the natural substance can be produced at
substantially lower cost.

Host cell line

The chosen host cell line was the DHFR⁻ mutant of the
permanent CHO (Chinese hamster ovary) line,
described by Urlaub et al (1980).

Vectors

The interferon gene

The starting material was the plasmid vector pBR 13,
which contains the genomic IFN-beta gene. A defined
portion of this genomic DNA was shortened by the
methods described by Maniatis et al (1982) and
recombined with an already-known vector (pSVd2-3).

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Promoter

Another important step was the selection of a
suitable promoter for regulation of expression.
Notwithstanding the possibility of using inducible
promoters, Hauser et al (1982) and Brinster et al
(1982), we preferred the strong constitutive
promoter of SV40 described by Mosthaf et al (1985),
which also includes an enhancer region. We came to
this decision after our own discouraging experience
with induction-dependent production processes
(negative feedback, cytotoxicity) on the one hand
and the possibilities offered by permanent
fermentation processes even for obligate adherent
cell cultures on the other hand. The construct,
described in further detail hereinafter, was called
pSVIFNAsu.

Selection

For the purpose of selection and amplification in
the direction towards a high-expressing cell line,
the DHFR gene was separately introduced into a
second plasmid pAdD26SV(A)-3. This was a means of
selection on the basis of IFN-beta expression, owing
to the interaction between the individual
adenopromoter and the enhancer region of the SV40
promoter (see above). In the present construct, as
expected, the common transfected DNA was
incorporated in close proximity.

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Transfection

The above expression vector, which contains
regulating sequences and the structure gene, and the
selection plasmid, which repairs the deficiency in
the cell line, were transfected together in an
experimentally determined optimum mixing ratio in
accordance with a calcium phosphate precipitation
method.

Amplification, cloning and cell bank

Interferon-expressing clones from the transfection
batch were amplified by a method first described by
Kaufman et al (1985) over a number of stages of
methotrexate concentration without intermediate
selection on high-producing colonies, and were then
cloned and after serial passages acquired a
sufficient number of cells, which were stored at a
stem cell bank in liquid nitrogen in accordance with
conventional methods of conservation.

Results

Production of the raw material

The cell line obtained by the method described and
given the laboratory designation BIC 8622 (BIC,
ECACC Entry No. 87040301) secreted after confluence
in conventional cell culture medium (modified
Eagle's MEM with Earle's salts) supplemented with
1.5% NCS or FCS in stationary culture in multitrays

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(NUNC) e.g. in the fermenter on micro-supports
(Cytodex III, Pharmacia), consisting of between 0.4
and 0.6 x 10^9 international units of IFN-beta per day
in a litre of culture supernatant.

Concentration

Concentration was via adsorption on a sulphopropyl
cation exchanger and subsequent immune sorption on
anti-IFN-beta sepharose (Celltech, Slough). Since
this immune sorption matrix contains monoclonal
antibodies against native fibroblast interferon,
adsorption and desorption under the conditions
optimised by the manufacturer for the immune binding
reaction, is an indication of the identity of the
synthetic molecule, which could be quantified by an
ELISA developed by using the same antibodies. This
was followed by a purification step consisting of
FPLC gel filtration for removing low-molecular and
high-molecular attendant materials.

Characterisation

Biological effect:

The bioassay developed for determination of natural
IFN-beta from FS-4 cells, using the antiviral
activity by measuring the inhibition of the
cytopathic effect of murine encephalomyocarditis
virus (EMCV) on an indicator cell (FS-4), modified
after Havell et al (1972), is also usable without
limitation for determination of IFN-beta from BIC

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cells.   The specific activity obtained by this test
and by protein determination after Lowry et al
(1951) is 2-3 x 10^6 IU which, within the limits of
accuracy of measurement, corresponds to the data
obtained for FS-4 interferon.

Immunological characterisation:

In addition to immune affinity chromatography used
for concentrating IFN-beta from BIC, a newly
developed ELISA and immune blotting techniques
showed substantial agreement in molecular properties
between natural and recombinant IFM-beta.   The
antibodies used were monoclonal mouse hybridomas
(MAK BO-2, Celltech) and polyclonal IgG from the
goat (Rega Institute, Leuwen).

Protein chemical characterisation:

The data obtained by immunological methods were
supplemented by amino acid analysis and sequencing
of 15 N-terminal amino acids.   No differences could
be shown between natural, Knight et al (1980) and
recombinant IFN-beta.   Investigation of the
carbohydrate component yielded substantially uniform
glycosylation for the recombinant IFN-beta, whereas
the natural IFN-beta carbohydrate was heterogeneous.

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Experimental part

Materials

E.coli K12 DH 1 (DSM)4079

CHO DUK DHFR⁻BF (ECACC Entry   This cell line originated
No. 87041401)   from mutagenesis and
                                 selection for absence of
                                 dihydrofolate reductase from
                                 the line CHO-K1 (ATCC CCL
                                 61), Urlaub et al (1980).

pBR 13: (DSM 4074P)   This plasmid is a derivative
                                 of the cloning plasmid pBR
                                 325, Bolivar et al (1977),
                                 having a single EcoRI
                                 interface into which a 1.83
                                 kb long DNA fragment was
                                 inserted. This fragment is
                                 contained in the cosmid pCos
                                 IFN-beta (Gross et al 1981),
                                 which itself is part of a
                                 cosmid bank of human
                                 placenta DNA.

PSVd2-3: (DSM 4075P)   This plasmid is a derivative
                                 of the cloning vector pAT
                                 153 which contains sequences
                                 from the eucaryotic DNA
                                 virus SV 40, Fiers et al.
                                 (1978). The starting
                                 plasmid for constructing the

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corresponding vector
plasmids was a construction
for expressing the mouse
dihydrofolate reductase
gene, Subramani et al.
(1981). The modification of
the expression vector pSV
used was produced by Dr D
Huylebroeck and is
substantially described in
the work by Fransen et al.
(1985).

pAdD26SV(A)-3: (DSM 4076P)   A plasmid derivative
originating from deletion of
a 1.1 kb long DNA fragment
from the cloning vector pBR
322, Lusky et al. (1981).
The plasmid also contains
the transcription initiation
signal (adenovirus major
late promoter) from the
eucaryotic DNA virus adeno
2, together with the cDNA of
the mouse dihydrofolate
reductase gene, the poly-
adenylation signal and a 200
bp long fragment with the
origin of replication from
SV 40, (Kaufman et al.
1982a).

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The expression system

The expression system used is based on a line of egg
stem cells from the Chinese hamster (CHO, ATCC CCL
61 CHO-K1) made deficient in the dihydrofolate
reductase enzyme by mutagenesis, Urlaub et al.
(1980). By incubation of a calcium phosphate
precipitate of DNA with these cells it is possible,
with very low frequency, to obtain absorption of the
DNA in the cells, Graham et al. (1973). The DNA
thus introduced into the cells is usually combined
and incorporated in the genome of the cell in
question, where it is propagated or changed
exclusively during cellular or DNA replication or
during the recombination processes occurring in the
cell genome, i.e. behaves like an integral natural
component of the cellular genome. In the special
case, an expression plasmid for expressing the human
IFN beta gene is incorporated in the CHO cells
together with a plasmid available in the mixture for
expression of the cDNA dihydrofolate reductase gene
from the mouse. The status of the expression
plasmid can be determined by the so-called Southern
Blot technique. To this end the cellular DNA is
cleaved with restriction endonucleases and separated
on an agarose gel in an electric field. The DNA
fragments are then bonded to a nylon filter membrane
and DNA is detected by DNA-DNA hybridisation with
radioactively labelled plasmid.

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Isolation of the interferon beta gene

The starting plasmid for isolation of DNA restriction fragments with the IFN beta gene was the plasmid pBR 13. In order to produce this plasmid, first the mRNA coding for IFN beta was transferred from FS 4-fibroblasts to cDNA and a cosmid pCOSIFN beta containing the IFN beta gene was isolated from a gene library (human placenta) by hybridisation with complementary genomic DNA. By cleaving the plasmid pBR 13 with the restriction endonuclease Eco RI, the fragment containing the IFN beta gene was first isolated from pCosIFNbeta and then was isolated, followed by further cleavage with the restriction endonucleases Hinc II or Nco I and Hind III, yielding smaller DNA pieces with the IFN-beta gene.

Construction of the expression plasmids

The plasmid for expression of the human IFN beta was constructed by using an expression vector called pSVd2-3. The functional components of this plasma ensure autonomous DNA replication in E.coli and selection of the E.coli cells by ampicillin (origin of replication of the transfer-negative plasmid pAT 153 and of the β-lactamase gene). The components determined for operation in eucaryotic cells consist of the promoter-enhancer region of the early genes of the virus SV 40 for initiation of transcription of the downstream genes and of the polyadenylation signal of the SV 40 virus for attaching a polyA

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sequence to the transcribed mRNA if a signal sequence of this kind is lacking in the gene used. Between the transcription initiation signal and the polyadenylation sequence there are number of synthetically produced recognition and cleavage sequences for restriction endonucleases for insertion of DNA for expression. The cleavage site of the nuclease Xba I was chosen for cloning the human IFN-beta DNA in this vector. To this end the vector was cleaved with Xba I. An Eco RI fragment with IFN beta gene was first isolated from the plasmid pBR 13, after which an Ncol – Hind III sub-fragment was isolated therefrom with IFN-beta gene. The overhanging single-stranded DNA ends produced by cleavage with the restriction endonucleases were filled by incubation with nucleoside triphosphates and the DNA polymer salt from E. coli. Synthetically produced polynucleotides containing the expression sequence of the restriction endonuclease Xba I were then enzymatically attached to the resulting smooth DNA ends of the fragment. Excess oligonucleotides were removed by incubation with the nuclease Xba I, which simultaneously resulted in overhanging single-stranded ends which were complementary with those of the vector DNA. The vector DNA and the IFN-beta DNA were enzymatically fitted together and introduced into E. coli cells. The cells containing IFN-beta DNA were identified by DNA-DNA hybridisation and used for purifying larger amounts of plasmid DNA. The plasmid DNA was characterised by cleavage with various restriction endonucleases and each plasmid

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was selected for further processing and called pSV
IFN Nco (DSM 4077 P), which conformed to
expectations. The plasmid pSV IFN Nco was then
partially cleaved with the enzyme Xba and the
fragments were separated by gel electrophoresis.
The bands corresponding to the linear plasmid were
dyed with the fluorescence dye ethidium bromide and
made visible in UV light and cut out and the DNA was
isolated. The isolated DNA was then incubated with
the nuclease Asu II and then enzymatically closed in
a ring. This procedure resulted in deletion of an
Xba I-Asu II fragment from the original expression
plasmid pSV IFN Nco. The resulting expression
plasmid was called pSV IFN Asu (DSN 4078 P) and used
for transfection of a CHO cell line deficient in
dihydrofolate reductase. Cells which had been
transfected with the expression plasmid pSV IFN Asu
and had incorporated their DNA in the cell genome
were made to form an mRNA consisting of a 60-
nucleotide portion of SV 40-specific sequences and
practically the authentic IFN beta mRNA attached
thereto. This mRNA was transferred by the protein
synthesis apparatus of the cells into an IFN-beta
protein which, in its amino-terminal region and its
amino acid composition, corresponded to the natural
IFN-beta protein from human fibroblast cells and was
glycocylated, in contrast to the proteins produced
in E. coli.

The DNA species and the manner of construction of
the expression plasmid pSV IFN Asu can be used to
derive the primary structure of the mRNA formed in

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eucaryotic cells which had incorporated this plasmid in their genome. Fig. 2 shows the structure of the expression plasmid with a detailed print-out of the relative cleavage sites. Transfer of the nucleotide sequence into an amino acid sequence by the genetic code yields the primary structure of the authentic IFN beta from human cells, Tavernier et al. (1984).

Transfection

The expression vector DNA was introduced into the CHO cell line substantially by a method described by Graham et al. (1973) and modified by Wigler (1979). Basically in this method, the cells are supplied with DNA precipitated from a solution containing CaPO4 coprecipitate. The DNA is then absorbed by the cells, probably by phagocytosis, and mainly lined up by unexplained mechanisms and incorporated in the cellular genome. This process is called transfection of eucaryotic cells with DNA. By combined precipitation of a vector for expression of the enzyme dihydrofolate reductase with the actual expression vector, when using dihydrofolate-deficient CHO cells, the transfection process can be used to select those cells which have absorbed DNA and incorporated it in their genome. This is done by cultivating the cells in a cell culture medium which lacks the components for nucleic acid synthesis.

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Amplification and selection

The culture supernatants of the thus-transfected and
selected cells were tested for beta-interferon
activity. The positive clones in this test were
subjected to a process for selectively multiplying
the DNA incorporated in the cells and increasing the
number of copies of the interferon gene in order to
increase the production of beta-interferon. This is
possible in principle by the gene-dose effect. The
method used for this purpose involves selection of
the beta interferon-producing cells in a culture
medium containing the enzyme inhibitor (+)
amethopterin. By means of this inhibitor, the
enzyme dihydrofolate reductase, whose gene has been
introduced into the CHO cells by transfection, is
inhibited in dependence on concentration. As a
result of this inhibition, those cells which have
increased the dihydrofolate reductase gene dose by
multiplication of the corresponding DNA have a
growth advantage. Since these DNA multiplication
processes usually involve larger portions and the
transfected DNA species are usually incorporated
close together in the genome of the cells, the dose
of the beta-interferon gene is simultaneously
increased. Stepwise increase of the (+)
amethopterin concentration in the culture medium and
intermediate selection and expansion phases result
in production of a cell mixture which delivers large
amounts of beta interferon to the culture medium.
This mixture, by dilute sowing of the cells in the
culture vessels and isolation of the cell colonies

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by means of metal cylinders, yielded pure lines of cells which likewise deliver large quantities of beta-interferon to the culture medium.

A producer cell library (= production cell library) was produced from these clones, called "BIC" cells, in accordance with the relevant guidelines, and portions of these cells were deposited in the "Public Health Laboratory Service, European Collection of Animal Cell Cultures (ECACC)", entry number 87040301.

Concentration

15 litres of culture supernatants with an average interferon content of 225 000 IU/ml were pooled by repeated harvesting in a 24-hour cycle from a confluent stationary culture of BIC taken from the production cell library and were together placed on a cation exchanger (sulphopropyl). 175 ml of eluate contained 3×10^9 IU, corresponding to an 83% yield. Subsequent immune sorption on BETA RESOLUTE R (Celltech) yielded an elution volume of 112 ml, containing 2.2×10^9 IU or 72% of the interferon with a specific activity of 3.8×10^8 IU/mg protein. Final gel filtration resulted in a total yield of 48%.

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Analysis

Biological and immunological characterisation

Highly purified preparations of recombinant BIC beta-IFN and natural FS 4-beta IFN were tested for their interferon content in an antiviral bioassay. In parallel therewith, the protein content was determined after Lowry. The natural material had a specific activity of about $2.7 - 3 \times 10^8$ international units (IU) per milligram protein and the recombinant material had an activity of $3 - 4 \times 10^8$ IU/mg. These values correspond to the data known from the literature for natural interferon. The specific activity in the prior art cannot at present be determined more precisely, owing to the relatively high inaccuracy of the bioassay.

The starting material and the concentrated highly-purified fractions were also subjected to an enzyme-coupled immune test (Elisa) after the antiviral bioassay.

In this test the plastic surface of microtitre plates is coated with polyclonal goat anti-IFN beta antibodies. The sample under test is then diluted. The beta-IFN bonds to the immobilised antibodies. In the next step this complex is reacted with a solution of monoclonal mouse anti-IFN beta antibodies. The resulting total complex is incubated with anti-mouse IgG antibodies previously conjugated with horseradish peroxidase. After

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removal of the non-bonded antibodies, the quantity
of bonded peroxidase and consequently the quantity
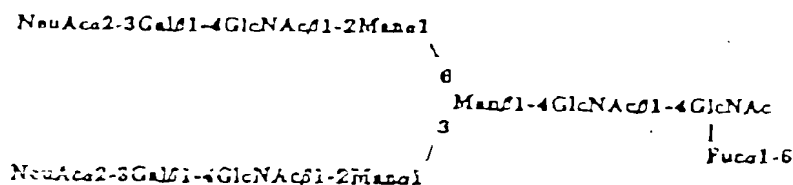of bonded beta-IFN can be determined by a colour
reaction.

This test was validated for natural beta-IFN
obtained from FS 4 fibroblasts. To this end
dilution series of samples having a known interferon
content were tested together with portions of the
international beta-IFN standard (NIH, G-023-902-
527). In all cases there was a close correlation
between the bioassay and the ELISA.

The concentrated, highly purified IFN preparations
obtained from the culture supernatants of BIC cells
uniformly corresponded in the ELISA to the values
obtained in parallel in the antiviral bioassay.
Aliquot portions of the international standard or of
preparations calibrated therewith were used for
standardisation in all cases. This therefore shows
that the antigenic properties relevant to the Elisa
were also identical between natural and recombinant
beta-IFN.

Sequence/amino acid composition

The recombinant IFN-beta produced and purified by
the method described was analysed with regard to its
amino acid composition and its amino-terminal
sequence.

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Partial sequencing up to the 15th amino acid was
effected by Edman decomposition in an automatic gas
phase sequencer (Messrs Applied Biosystems, Type 470
A). The resulting phenyl hydantoin derivatives were
separated on a PTH-C80 matrix and then detected.
The result agrees with the data published by Lawn et
al (1981), Ohno et al (1981) and Derynck et al
(1980). Analysis of the amino acid composition,
wherein the PTH amino acids were subjected to the
same methods of separation after hydrolysis, show
that the values obtained by Knight et al (1980) for
FS-4 were also true for the IFN-beta from CHO.

Glycosylation

The polypeptide obtained after deglycosylation or
after inhibition of glycosylation with tunicamycin
had the same electrophoretic properties under
denaturing and non-denaturing conditions as the
similarly obtained IFN beta from FS-4. The
oligosaccharides liberated by glycopeptidase F after
sequential decomposition by exoglycosidase, were
subjected to methylation analysis and FAB mass
spectrometry. It was shown that 95 ± 5% of the
carbohydrate side chains were biantennary complex
type and had the following structure:

NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1
                                      \
                                       6
                                        Manβ1-4GlcNAcβ1-4GlcNAc
                                       3 |
                                      /   Fucα1-6
NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1

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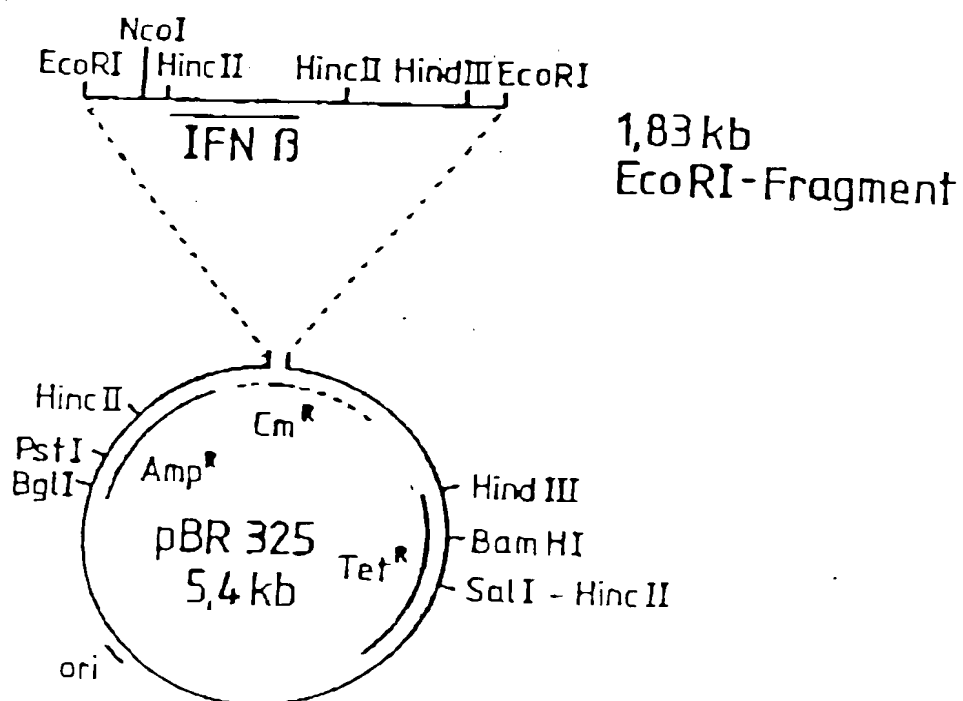
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Claims

1. Recombinant cell line BIC 8622 (ECACC 87040301).

2. Process for the constitutive preparation of human IFN-β1, wherein a recombinant cell line according to claim 1 is cultured and the IFN-β1 is isolated from the cell supernatant.

3. Process according to claim 2, further comprising the use of the human IFN-β1 obtained for the preparation of a medicament.

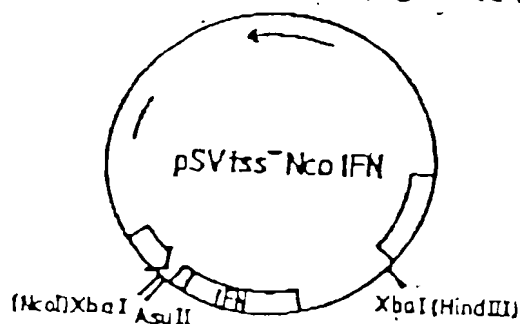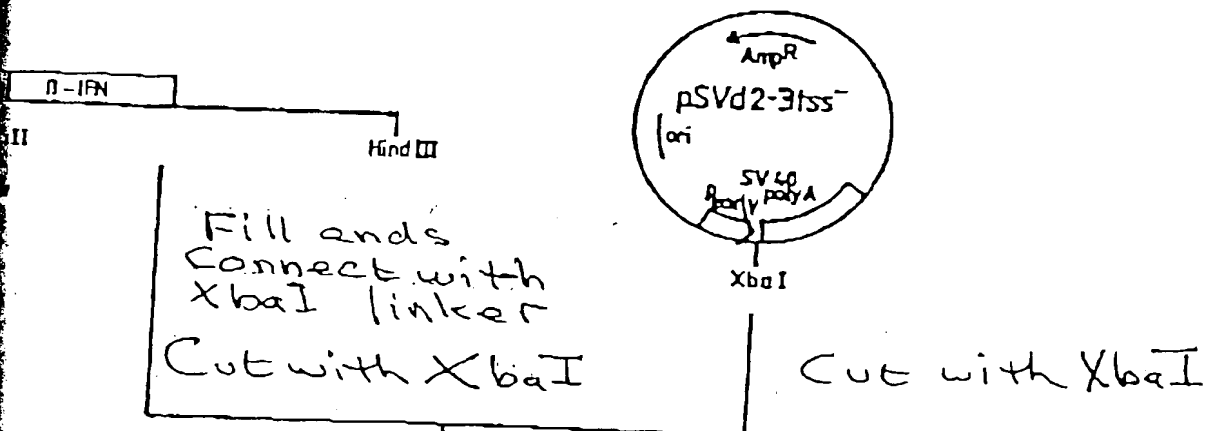4. Process according to claim 3 for the preparation of an agent for the treatment of virus infections.

EP 0 287 075 B1

pBR 13

NcoI

EcoRI | HincII   HincII HindIII EcoRI

IFN ß

1,83 kb
EcoRI-Fragment

HincII
PstI
BglI

Amp^R Cm^R

pBR 325
5,4 kb   Tet^R

ori

Hind III
Bam HI
Sal I - Hinc II

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